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Community Reference Laboratory for Feed Additives

CRL Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**EFSA-Q-2007-120** **FAD-2007-0020**
Product name:	**Econase XT L & P**
Active Substance(s):	**Endo-1,4-β-xylanase (EC 3.2.1.8)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
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Date:	**13/02/2008**
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Date:	**14/02/2008**

EXECUTIVE SUMMARY

The current application authorisation is sought for *Econase XT L & P* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Econase XT L&P* as a digestibility enhancer for chickens for fattening/reared for laying; turkey for fattening/reared for breeding; and for piglets (weaned). The product is intended to be marketed as solid (*Econase XT P*) and as liquid (*Econase XT L*) formulations.

The active agent of *Econase XT L&P* is endo-1,4-β-*xylanase* produced by a strain of *Trichoderma reesei* (CBS 114044). The enzymatic activity is expressed in *xylanase* unit (BXU) where 1 BXU is the amount of endo-1,4-β-*xylanase* that liberates 1 nmol xylose from birch xylan per second at pH 5.3 and 50^{o}C. The solid product (*Econase XT P*) has a target activity of 4 000 000 BXU/g. It is intended to be mixed into *premixtures* and/or *feedingstuffs* to provide an enzyme activity range of 6 000 to 24 000 BXU/kg *feedingstuffs*. The liquid product (*Econase XT L*) has an enzyme activity of 400 000 BXU/g, and is sprayed directly onto the post-pelleted feed to obtain an enzyme activity range of 6 000 to 24 000 BXU/kg *feedingstuffs*.

An absolute colorimetric method based on the formation of reducing sugar reacted with dinitrosalysilic acid (DNS) is in-house validated for the determination of the activity of endo-1,4-β-*xylanase* in the *feed additive* and *premixtures*. The following performance characteristics were obtained for liquid and powder *feed additives* and for turkey and broiler *premixtures*: recovery greater than 85% and relative standard deviations for repeatability and intermediate precision ranging from 2.0 to 6.0% and 4.0 to 11.0%, respectively. However, low recovery rate (60%) and high relative standard deviations for repeatability and intermediate precision (ca. 24%) were reported for the piglet premixture.

An analytical method based on the measurement of the rate of release of water soluble dyed fragments by endo-1,4-ß-*xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet" is in-house validated for the determination of the activity of endo-1,4-β-*xylanase* in the *feedingstuffs*. The following performance characteristics were obtained for turkey and broiler *feedingstuffs*: recovery of 104% and relative standard deviations for repeatability and intermediate precision ranging from 4 to 7% and 5 to 7%,

respectively. Insufficient experimental data was provided to establish the validity of analytical method for the determination of active substance (*xylanase*) in the piglet *feedingstuffs*.

Based on acceptable performance characteristics, the two proposed methods are considered suitable for determination of *xylanase* activity - in *feed additives*, *premixtures* and *feedingstuffs* for turkeys and broilers (not for piglets) - for official control purposes in the frame of authorisation.

Further testing or validation for the methods determining xylanase activity - in *feed additives*, *premixtures* and *feedingstuffs* for turkeys and broilers is not considered necessary.

KEYWORDS

Econase XT L&P, endo-1,4 β-*xylanase*, *Trichoderma reesei*, digestibility enhancer, chickens for fattening/reared for laying, turkey for fattening/reared for breeding, piglets (weaned).

1. BACKGROUND

Econase XT L&P is a product for which authorisation as feed additive is sought under the category ‘zootechnical additives’, functional groups ‘digestibility enhancers’, according to Annex I of Regulation (EC) No 1831/2003 [1]. It contains endo-1,4-β *xylanase* (EC 3.2.1.8) as the active agent [2], produced by a strain *Trichoderma reesei* (CBS 114044), which has been deposited at the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands [3]. The activity of endo-1,4-β *xylanase* is expressed as *xylanase* unit of BXU. According to the applicant, one BXU is the amount of endo-1,4-β-*xylanase* that liberates 1 nmol reducing sugars (xylose equivalent) from birch xylan per second at pH 5.3 and 50^{o}C [6]. The product is intended to be marketed in two forms [4]:

- *Econase XT P* is a solid formulation with a target endo-1,4-β *xylanase* activity of 4 000 000 BXU/g, to be mixed with the feedingstuffs before pelleting;
- *Econase XT L* is a liquid formulation with a target endo-1,4 β-*xylanase* activity of 400 000 BXU/g, to be sprayed onto the feed during post-pelleting.

Econase XT L&P is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a minimum enzyme activity levels of 8 000 BXU/kg in *feedingstuffs* for chickens for fattening/reared for laying and of 6 000 BXU/kg in *feedingstuffs* for turkey for fattening/reared for breeding and of 16 000 BXU/kg in *feedingstuffs* for piglets (weaned), respectively [5]. The applicant proposed the following as recommended dosages: 8 000 to 24 000 BXU/kg in *feedingstuffs* for chickens for fattening/reared for laying; 6 000 to 24 000 BXU/kg in *feedingstuffs* for turkey for fattening/reared for breeding and 6 000 to 16 000 BXU/kg in *feedingstuffs* for piglets (weaned), respectively [6].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis, submitted in connection with *Econase XT L&P*, (EFSA-Q-2007-120), and their suitability to be used for official controls in the frame of authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and qualitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of impurities in the *additive* (e.g. arsenic and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [7].

Description of the analytical methods for the determination of the active agent in the feed additive, premixtures and feedingstuffs

Method 1: Determination of activity of endo-1,4-β-*xylanase* in the *feed additive* and *premixtures* [8]

The applicant proposes an absolute colorimetric method (Method 1) based on the formation of reducing sugar reacted with dinitrosalysilic acid (DNS), where the colour change is proportional to xylose equivalents measured at 540 nm. Approximately 0.5 g of feed additive as liquid/powder formulation is added with a 25 ml of citrate buffer (0.05M, pH 5.3) and stirred at room temperature for 30 minutes (only for dry formulation). 5 g sample of *premixture* is added with 40 ml citrate buffer containing 2% EDTA, stirred for 30 minutes and centrifuged for 10 minutes. 1.8 ml substrate solution containing 1% birch xylan is added to the suitably diluted samples, and incubated at 50 ^{o}C for 5 minutes. After exactly 5 minutes, 3.0 ml DNS solution is added. The blank sample undergoes a similar procedure, where the enzyme solution is added after the reaction is stopped by DNS. The absorbance is then measured against the enzyme blank at 540 nm. A four points calibration curve is constructed using xylose (Merck 8689) standard solution for the determination of unknown enzyme activity. Limits of detection and quantification (LOD and LOQ) of 36 BXU/g and 68 BXU/g, respectively, were obtained. These values are far below the declared levels of *xylanase* activity in both *feed additive* formulations. The performance characteristics of Method 1 were established by an in-house validation study. Recovery rate (%), relative standard deviation for repeatability (RSD_r%) and relative standard deviation for intermediate precision (RSD_R%) are reported in Table 1.

Table 1: Performance characteristics of the method 1 [9].

The product/matrix	Feed additives		Premixtures		
Formulation	Liquid	Powder	Turkey	Broiler	Piglet
Activity, BXU/g	400-1500 x10^3	4000 x10^3	3-120 x10^3	3-6 x10^3	3-6 x10^3
Recovery, %	100	85	86	104	< 80
Repeatability	2,1%	3,7%	2,6%	6,0%	24%
Intermediate precision	4,1%	7,2%	5,0%	11%	24%

Method 1 determining the *xylanase* activity in the *feed additive* and in the *premixtures* shows acceptable performance characteristics (Table 1). However, for the piglets premixtures, the measured *xylanase* activities were highly scattered as shown by the high relative standard deviations of 24%. The corresponding low recovery rate (< 80%) may be attributed to the

interference of highly concentrated metal ions present in this premixture. The applicant was requested to submit additional experimental data to demonstrate the suitability of the method for the determination of *xylanase* activity in *premixtures* for piglets. No satisfactory data [10] was further provided.

Based on the above mentioned performance characteristics Method 1 is considered suitable for the determination of *xylanase* activity in *feed additives*, and in *premixtures* for turkeys and broilers (not for piglets) - for official control purposes in the frame of the authorisation.

Method 2: Determination of *xylanase* activity in the *feedingstuffs* [11].

An analytical method (Method 2) is proposed based on the measurement of the rate of release of water soluble dyed fragments by endo-1,4-ß-*xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet" available from Megazyme. 2.5 g of ground feed samples with *xylanase* activity is added with 20 ml acetate buffer solution (0.05 M, pH 5.5) and stirred for 30 minutes at room temperature. Sample extract is then incubated with one "Xylazyme AX" tablet for 5 minutes at 50^{o}C. The reaction is stopped by adding 5.0 ml Trizma base. The absorbance is then measured against an enzyme blank at 590 nm. The blank is prepared by adding Trizma Base to the enzyme solution before the addition of "Xylazyme AX tablet", with no incubation at 50^{o}C. The external calibration curve is obtained when assaying a series of feed additive dilutions containing known *xylanase* activities determined by Method 1, described earlier. The same form of feed additive (liquid or powder) used for the preparation of feedingstuffs should be used for the calibration, and therefore made available to the official control laboratories.

The applicant investigated the limit of detection (LOD) and the limit of quantification (LOQ) analysing of two blank feedingstuffs containing respectively 750 and 2500 BXU/kg of naturally occurring (endogenous) *xylanase*. A total of 36 replicate samples per blank were analysed to derive a standard deviation (Std) of 250 BXU/kg. Applying the following formulas: LOD = Yo+3*Std and LOQ = Yo+10*Std, they derive two sets of LOD and LOQs - depending of the endogenous *xylanase* contents.

The CRL-FA considers that Method 2 does not distinguish between "endogenous " and "added" xylanase, but allows the determination of total *xylanase*. It is therefore assumed

that only one set of (LOD, LOQ) characterises Method 2. The CRL-FA computed this unique set after "blank" correction, to obtain LOD = 3* Std = 750 BXU/kg and LOQ = 10*Std = 2500 BXU/kg.

The precision of Method 2 was established by an in-house validated study, for *xylanase* activity ranging from 16 000 to 240 000 BXU/kg. Insufficient experimental data was reported for lower activities, down to 6 000 BXU/kg. Recovery rate (%), relative standard deviation for repeatability (RSD_r%) and relative standard deviation for intermediate precision (RSD_R%) are reported in Table 2.

Table 2: Performance characteristics of method 2 [9]

Feedingstuffs	Broiler	Turkey		
Xylanase, BXU/kg	24 000-109 000	16 000	24 000	240 000
Recovery, %	104	101		
Repeatability	4,4%	3,8%	6,7%	5,4%
Intermediate precision	n.a	5,4%	7,3%	6,7%

n.a: not available.

Method 2 shows acceptable performance characteristics for the target levels ranging from 16 000 to 240 000 BXU *xylanase* per kg *feedingstuffs* for the animals species of broilers and turkeys; it is further assumed to be suitable at lower *xylanase* activity levels, down to 6 000 BXU/kg. Since the validation experiment for the piglet *premixtures* indicated an adverse effect of this matrix on the performance characteristics of Method 1, it was assumed that the dossier contained more validation results of Method 2 for the piglet *feedingstuffs*. However, the applicant provided insufficient analytical data demonstrating the suitability of Method 2 for the determination of *xylanase* activity in *feedingstuffs* for piglets.

Based on the above mentioned performance characteristics Method 2 is considered suitable for the determination of *xylanase* activity in *feedingstuffs* for turkeys and broilers (not for piglets) - for official control purposes in the frame of the authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of *Econase XT L&P* authorisation, the CRL recommends the two proposed methods for determination of *xylanase* activity - in *feed additives, premixtures* and *feedingstuffs* for turkeys and broilers (not for piglets) - for official control purposes.

Further testing or validation for the methods determining *xylanase* activity - in *feed additives, premixtures* and *feedingstuffs* for turkeys and broilers is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Characterisation of the active substance in the *feedingstuffs*:

Colorimetric method measuring water soluble dye released by the enzyme from azurine crosslinked wheat arabinoxylan substrate.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Econase XT L&P* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Reference SANCO/D/2 Forw. Appl. 1831/16-2007.

[2] * Technical Dossier_Vol1_DOSSIER ECONASE XT: Section II, Subject 2, Item 1.3.

[3] * Technical Dossier_Vol1_ DOSSIER ECONASE XT: Section II, Enclosure 08.

[4] * Technical Dossier_Vol1_DOSSIER ECONASE XT: Section II, Subject 2, Item 1.5.

[5] * Application: Annex III_XTL/P, Proposal of Register entry.

[6] * Technical Dossier_Vol1_DOSSIER ECONASE XT: Section II, Subject 2, Item 5.1.

[7] COMMISSION REGULATION (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards Community reference laboratories, Official Journal of the European Union L 136

[8] *Supplementary information, enclosure 1 (xylanase assay method).

[9] * Technical Dossier_Vol1_DOSSIER ECONASE XT: Enclosure 36 (validation study).

[10] *Supplementary information, enclosure 3-extraction test.

[11] * Technical Dossier_Vol1_DOSSIER ECONASE XT: Enclosure 38 (feed method).

*Refers to Dossier number: FAD-2007-0020

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium.

8. ACKNOWLEDGEMENTS

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- Plantedirektoratets Laboratorium, Lyngby, Denmark.
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- National Research Institute of Animal Production in Krakow, National Feed Laboratory, Lublin, Poland.
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- National Veterinary Research Institute, Pulawy, Poland.